Ministry of Economic Development
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Intellectual Property Office of New Zealand

PCT/NZ2004/000255

CERTIFICATE

This certificate is issued in support of an application for Patent registration in a country outside New Zealand pursuant to the Patents Act 1953 and the Regulations thereunder.

I hereby certify that annexed is a true copy of the Provisional Specification as filed on 17 October 2003 with an application for Letters Patent number 528966 made by ROGER MICHAEL ECCLES; ALEKSANDRA MURATOVSKA; ROBIN A J SMITH and MICHAEL PATRICK MURPHY.

I further certify that pursuant to a claim under Section 24(1) of the Patents Act 1953, a direction was given that the application proceed in the name of UNIVERSITY OF OTAGO.

Dated 8 November 2004.

Neville Harris
Commissioner of Patents, Trade Marks and Designs

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NEW ZEALAND PATENTS ACT 1953

No:
Date:

PROVISIONAL SPECIFICATION

TRIPHENYLPHOSPHONIUM PEPTIDE NUCLEIC ACID CONJUGATES AND USES THEREOF

We, ROGER MICHAEL ECCLES OF 72 Cherry Drive, Mosgiel, New Zealand, ALEKSANDRA MURATOVSKA of 68 Petersfield Mansions, Mill Road, Cambridge CB1 1BB, United Kingdom, ROBIN A J SMITH of 20 Lynn St, Dunedin, New Zealand and MICHAEL PATRICK MURPHY of 74 Blinco Grove, Cambridge, CB1 TTS, United Kingdom, do hereby declare the invention for which we pray that a patent may be granted to us, and the method by which it is to be performed, to be particularly described in and by the following statement:

TRIPHENYLPHOSPHONIUM PEPTIDE NUCLEIC ACID CONJUGATES AND USES THEREOF

TECHNICAL FIELD

The invention relates to triphenylphosphonium (TPP)-peptide nucleic acid (PNA) conjugates and uses thereof.

BACKGROUND OF THE INVENTION

Peptide nucleic acid oligomers (PNAs) are DNA analogues that have an uncharged polyamide backbone comprised of N-(2-aminoethyl) glycine units that replace the deoxyribose-phosphate linkages in DNA (Egholm *et al.*, 1993). As this modification to the backbone does not alter the spacing of the bases related to DNA and RNA, PNAs can be designed to be complementary to a particular mRNA transcript permitting the antisense oligomer to undergo Watson-Crick hybridisation with its target (Egholm *et al.*, 1993). This results in mRNA inactivation through steric blocking of the ribosome complex and consequently, specific inhibition of the synthesis of a particular protein product. As such, PNAs can be used as tools to manipulate gene expression and may have application as therapies for a range of diseases.

However, a disadvantage of the application of PNAs as antisense agents is their low rate of membrane permeation (Eriksson *et al.*, 1996).

Several attempts have been made to circumvent this difficulty. For example, PNAs have been conjugated to cell penetrating peptides such as penetratin, Tat and transportan for delivery to the cytoplasm or nucleus of cells (Simmons *et al.*, 1997;

Eriksson *et al.*, 2001). However, the synthesis of these peptides is expensive and complex.

PNAs have also been encapsulated in cationic liposomes to improve cellular uptake (Ljungstrom *et al.*, 1999). However, this method is very dependent on the cell type and also dependent on the PNA sequence, and cellular uptake is quite slow. In addition, liposomes themselves induce a stress-response in cells and are cytotoxic at high concentrations.

Therefore, there is still the need for a simple and effective way to deliver PNA oligomers across the plasma membrane to the cytoplasm of the cell.

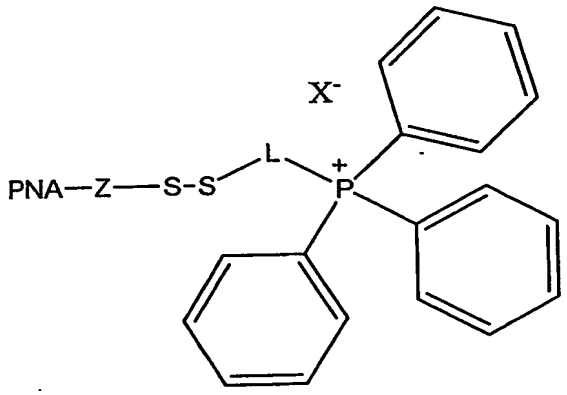
It has been shown that PNAs conjugated to a lipophilic cation easily pass through lipid bilayers. For example, the phosphonium cation catalyses the uptake of PNA through lipid bilayers as even the relatively small membrane potential of the plasma membrane 30-60 mV (negative inside) helps deliver cargo conjugated to lipophilic cations into the cytoplasm.

However, the large membrane potential (-150 to -170 mV) across the mitochondrial membrane causes lipophilic cation-PNA conjugates to selectively localise to mitochondria within cells (Muratovska *et. al.*, 2001; WO 99/26954). Consequently, techniques utilising known lipophilic cation-PNA conjugates are limited to the selective manipulation of mitochondrial DNA.

Accordingly, it is an object of the present invention to provide lipophilic cation conjugates useful for transporting PNA oligomers into cells without being taken into the mitochondria and/or to provide the public with a useful choice.

SUMMARY OF THE INVENTION

In one aspect, the invention provides a conjugate of formula I



wherein L is a linker group, S-Z is a thiol-containing attachment group, X^- is an optional anion, and PNA is a peptide nucleic acid.

Preferably, the linker group is (C_1 – C_{30}) alkylene or substituted (C_1 – C_{30}) alkylene. More preferably the linker group is (C_3 – C_{10}) alkylene. Most preferably, the linker group is butylene.

Preferably Z is selected so that S-Z is a cysteinyl, homocysteinyl or an aminothiol compound attached to a suitable linking group for linking to the PNA residue.

More preferably Z is selected so that S-Z is a cysteinyl, homocysteinyl or an aminothiol compound attached to an 8-amino-3,6-dioxanoic acid residue.

The phenyl groups of the triphenylphosphonium moiety may be optionally substituted with alkyl groups or any other group provided that the conjugate remains hydrophobic enough to transfer across the cell membrane.

The anion X^- is optionally present as required for overall electrical neutrality.

Preferably, the anion is an inorganic anion derived from hydrochloric, hydrobromic, hydroiodic, sulfuric, sulfurous, nitric, nitrous, phosphoric or phosphorous acid, or from an alkylsulfonic or an arylsulfonic acid.

More preferably, the anion is a halo anion, most preferably bromide.

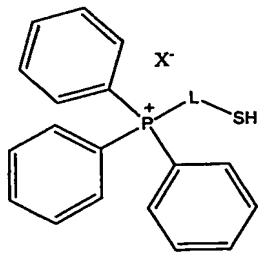
Preferably, the PNA is attached to a molecular tag or reporter molecule such as an affinity label (for example, biotin (bio), streptavidin etc) through a spacer group such as one or more 8-amino-3,6-dioxanoic acid residues.

More preferably the reporter is a fluorophore (such as for example Cy3, Cy5 and Cy2), most preferably, fluroscein (flu).

Preferred PNA oligomers are those targeting a unique region in both the human and mouse *PAX2* mRNA.

In another aspect the invention provides a method of synthesizing TPP-PNA conjugates according to Formula I comprising:

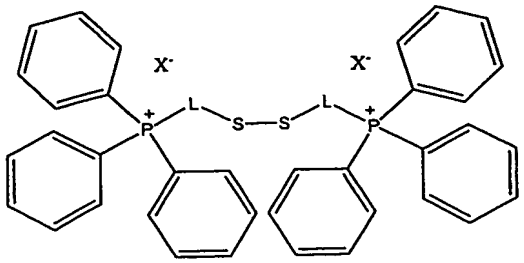
(a) incubating a compound of Formula II, wherein L and X are defined as above,



II

with an oxidant, to form the disulphide compound of Formula III

III



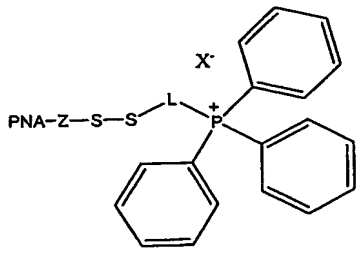
(b) reacting the compound of Formula III from step (a) with a compound of Formula IV

IV

PNA—Z——SH

wherein Z and PNA are defined as above, and wherein the compound of Formula IV has been preincubated with a non-thiol containing reducing agent, to form the TPP-PNA conjugate of Formula I.

I



In a further aspect, the invention provides a pharmaceutical composition comprising a therapeutically effective amount of a compound of Formula I in combination with one or more pharmaceutically acceptable excipients, carriers or diluents.

In a yet further aspect, the invention provides a use of a compound of Formula I in the preparation of a medicament for the treatment of a disease or disorder that can be at least in part alleviated by antisense therapy.

The invention also provides a method of treating a patient with a disease or disorder that is susceptible to antisense therapy, which comprises the step of administering to said patient, a therapeutically effective amount of a compound of Formula I or a composition of the invention.

The invention further provides a method of targeting PNA oligomers to the cytoplasm of a cell using a compound of Formula I, said method comprising delivering the PNA oligomers across the plasma membrane, without promoting selective aggregation in the mitochondria of said cell.

The invention further provides a method for modifying gene expression by administering a compound of Formula I to a cell.

DESCRIPTION OF THE DRAWINGS

In particular, a better understanding of the invention will be gained with reference to the following figures in which:

Figure 1 is a schematic representation of uptake of disulphide linked TPP-PNA conjugates into a cell.

Figure 2 shows the synthesis of a disulphide linked TPP-PNA conjugate

Figure 3 shows the purification and characterization of disulphide linked TPP-PNA conjugates by RP HPLC.

Figure 4 shows the characterization of disulphide linked TPP-PNA conjugates using a MALDI ToF mass spectroscopy analysis (4A and 4B) and immunoblotting (4C).

Figure 5a shows uptake of disulphide linked TPP-PNA conjugates by 143B osteosarcoma.

Figure 5b shows uptake of disulphide linked TPP-PNA conjugates by P388 cells.

Figure 6 shows the localization of the disulphide linked TPP-PNA conjugates visualized with confocal immunofluorescent microscopy.

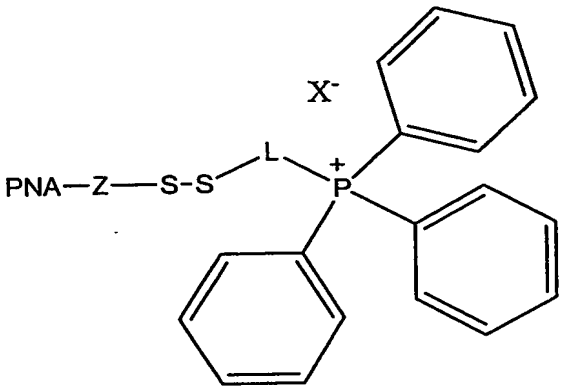
Figure 7 shows a western blot of P388 cells treated with 1μM anti-*PAX2* TPP-fluPNA treated with 1 μM anti-*PAX2* TPP-fluPNA and with media only.

DETAILED DESCRIPTION OF THE INVENTION

In one aspect the present invention is directed towards methods of targeting PNAs to the cytoplasm of a cell using a lipophilic cation modified to dissociate from the PNA in the cytoplasm thereby preventing selective accumulation in the mitochondria.

The applicants have unexpectedly found that this can be achieved by conjugating a PNA oligomer to the phosphonium cation thiobutyltriphenylphosphonium (TBTP) (Burns *et al.*, 1995) via a disulfide bond that is stable in the oxidising extracellular environment but is labile in the reducing cytoplasmic milieu (Fig.1). Once this TPP-PNA conjugate crosses the plasma membrane driven by the membrane potential, the disulfide bond is reduced by the cytoplamic GSH pool. The PNA is then released into the cytoplasm, while the dissociated lipophilic cation accumulates into mitochondria.

In one aspect the invention therefore provides a conjugate of Formula I



wherein L is a linker group, S-Z is a thiol-containing attachment group, X^- is an optional anion and PNA is a peptide nucleic acid group.

The linker group may be any group which joins the triphenylphosphonium cation moiety to the PNA moiety, and enables the two moieties to remain bonded together when crossing the plasma membrane.

Typically, the group will be an alkylene group. The term "alkylene" as used herein, pertains to a bidentate moiety obtained by removing two hydrogen atoms, either both from the same carbon atom, or one from each of two different carbon atoms, of a hydrocarbon compound having from 1 to 30 carbon atoms, preferably 3 to 10, more preferably 4, which may be aliphatic or alicyclic, and which may be saturated, partially unsaturated, or fully unsaturated. Thus, the term "alkylene" includes the sub-classes alkenylene, alkynylene, and cycloalkylene.

Alternatively, the linking group may contain one or more heteroatoms such as O or S.

The linking group may be substituted by substituent groups that increase the solubility of the molecule, increase the uptake of the molecule across the plasma membrane, or decrease the rate of degradation of the molecule *in vivo*. In particular, the linking group may be substituted by: hydroxyl, thio, amino, carboxy, amido groups or groups derived from sugars or sugar derivatives.

The anion comprises a suitable inorganic or organic anion known in the art and is present when required for overall electrical neutrality.

Examples of suitable inorganic anions include, but are not limited to, those derived from hydrochloric, hydrobromic, hydroiodic, sulfuric, sulfurous, nitric, nitrous, phosphoric or phosphorous acid or from an alkylsulfonic or an arylsulfonic acid.

Examples of suitable organic anions include, but are not limited to, those derived from the following organic acids: 2-acetyoxybenzoic, acetic, ascorbic, aspartic, benzoic, camphorsulfonic, cinnamic, citric, edetic, ethanedisulfonic, ethanesulfonic, fumaric, glucheptonic, gluconic, glutamic, glycolic, hydroxymaleic, carboxylic, isethionic, lactic, lactobionic, lauric, maleic, malic, methanesulfonic, mucic, oleic, oxalic, palmitic, pamoic, pantothenic, phenylacetic, phenylsulfonic, propionic, pyuvic, salicylic, stearic, succinic, sulfanilic, tartaric, toluenesulfonic, and valeric.

All are generally recognized as pharmaceutically acceptable salts.

More preferably, the inorganic anions are preferred, in particular, the halo anions, especially the bromide anion.

The spacer group S-Z may be any group containing a free thiol functionality to allow the PNA to bond to the TPP moiety. Preferably S-Z is a cysteinyl, homocysteinyl or aminothiol compound linked to 8-amino-3,6-dioxanoic acid.

PNA oligomers can be obtained commercially and may already incorporate the attachment group S-Z at the 3' end, and other spacer groups at the 5' end of the oligomer as required for reporter molecules.

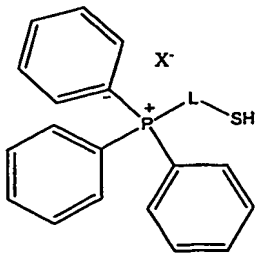
The spacer groups at the 5' end may be the same as Z or any other suitable spacer group.

The optional reporter molecule enables the conjugate to be easily detected with high sensitivity, and is compatible with the biological function of the conjugate. Such moieties include but are not restricted to a range of molecular tags such as affinity labels (for example, biotin, streptavidin etc) that can be attached to PNAs or more preferably fluorophores (such as for example Cy3, Cy5 and Cy2), or most preferably, fluroscein. The presence of a reporter molecule allows the sensitive detection of the conjugates by fluorescence microscopy, immunoblotting or affinity purification. Detection of the conjugates may be important in some applications such as trace localization/distribution in an organism/tissue, for example, to assess whether the conjugate crosses the blood-brain barrier or the placenta. In other applications, for example, treatment of disease, the reporter molecule may not be necessary.

Preferred PNA oligomers are those targeting a unique region in both the human and mouse *PAX2* mRNA.

In another aspect the invention provides a method of synthesizing TPP-PNA conjugates according to Formula I comprising:

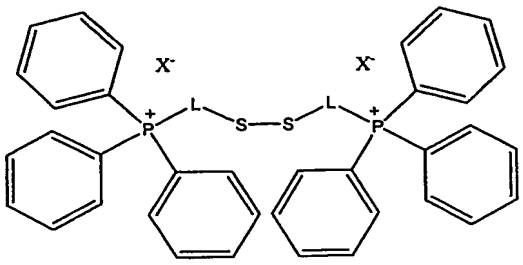
(a) incubating a compound of Formula II, wherein L and X are defined as above,



II

with an oxidant, to form the disulfide compound of Formula III

III



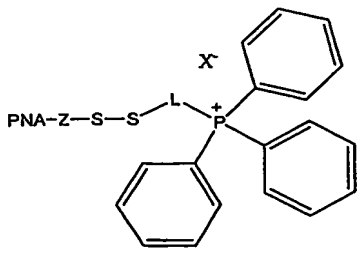
(b) reacting the compound of Formula III from step (a) with a compound of Formula IV

IV

HS—Z——PNA

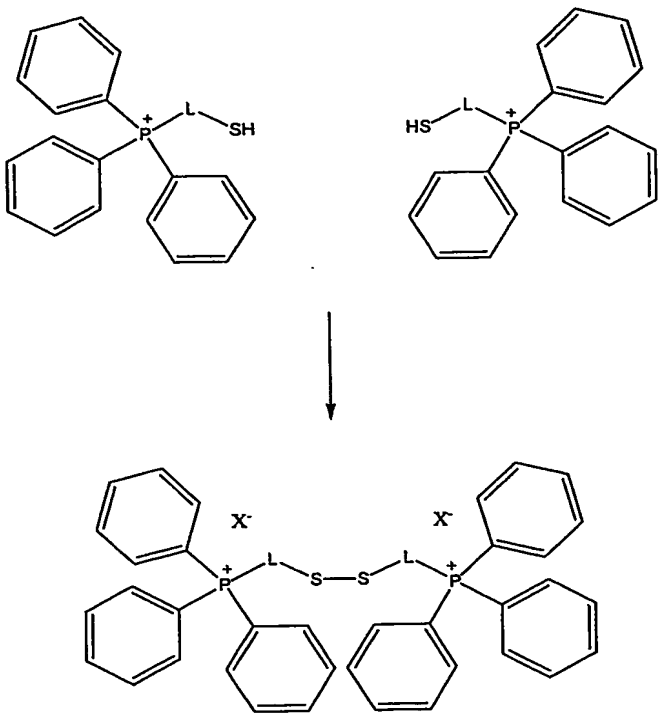
wherein Z and PNA are defined as above, and wherein the compound of Forumula IV has been preincubated with a non-thiol containaining reducing agent, to form the TPP-PNA conjugate of Formula I.

I



The synthesis of the TPP-PNA conjugate can be carried out in two stages as illustrated by the example shown in Fig 2. The first stage is the synthesis of a bistriphenylphosphonium disulfide (bisL-TTP) (Scheme 1).

Scheme 1

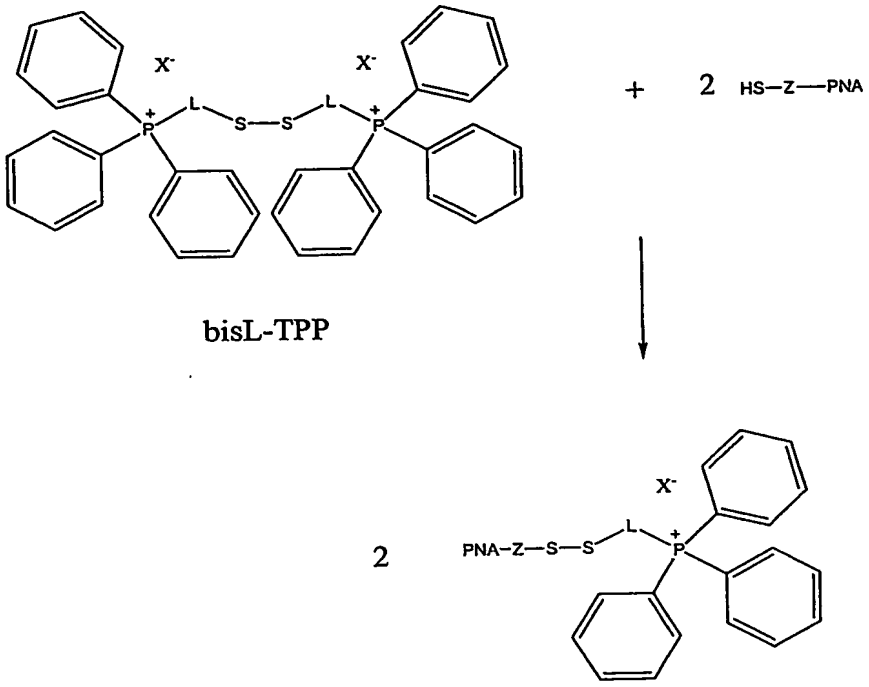


bisL-TPP

The thiol-linker-TPP compound is generated by base hydrolysis of acylated thiol-TPP. The reaction is quenched with acid HX which provides the counter-ion X^-

In the second stage the bisL-TPP is reacted with a thiol-containing PNA oligomer to form the TPP-PNA conjugate (Scheme 2).

Scheme 2



The PNA oligomer is first preincubated with a non-thiol containing reducing agent such as Tris[2-carboxyethyl]phosphine hydrochloride.

The cysteine residue of the thiol-linked PNA conjugate forms a disulfide linkage with the thiol-linked TPP to make a TPP-PNA conjugate. The coupling efficiency of the thiol containing compounds can be monitored by assaying the free thiol groups.

One of the advantages of this synthetic strategy is that the only major chemical species that are present at the end of the reaction are the TPP-PNA product and an excess of unreacted bisL-TPP.

In another aspect, the invention provides a pharmaceutical composition comprising a therapeutically effective amount of a compound of formula I in combination with one or more pharmaceutically acceptable excipients, carriers or dilutents.

Suitable excipients, carriers and diluents can be found in standard pharmaceutical texts. See, for example, Handbook for Pharmaceutical Additives, 2nd Edition (eds. M. Ash

and I. Ash), 2001 (Synapse Information Resources, Inc., Endicott, New York, USA) and Remington's Pharmaceutical Science, (ed. A. L. Gennaro) 2000 (Lippincott, Williams and Wilkins, Philadelphia, USA) which are incorporated herein by reference.

The term "pharmaceutically acceptable" as used herein pertains to compounds, ingredients, materials, compositions, dosage forms, etc., which are within the scope of sound medical judgment, suitable for use in contact with the tissues of the subject in question (e.g. human) without excessive toxicity, irritation, allergic response, or other problem or complication, commensurate with a reasonable benefit/risk ratio. Each carrier, diluent, exipient, etc., must also be "acceptable" in the sense of being compatible with the other ingredients of the formulation.

In a further aspect, the invention provides a use of a compound of formula I in the preparation of a medicament for the treatment of a disease or disorder that can be at least in part alleviated by antisense therapy.

The medicament may be prepared by any methods well known in the art of pharmacy. Such methods include the step of bringing into association the active compound with a carrier which constitutes one or more accessory ingredients.

In a yet further aspect, the invention provides a method of treating a patient with a disease or disorder that is susceptible to antisense therapy, which comprises the step of administering to said patient, a therapeutically effective amount of a compound of formula I or a composition of the invention.

The compound or pharmaceutical composition may be administered to a subject by any convenient route of administration, for example systemically/peripherally or topically (i.e., at the site of the desired action).

The term "treatment" as used herein in the context of treating a condition, pertains generally to treatment and therapy, whether of human or animal, in which some desired therapeutic effect is achieved, for example, the inhibition of progress of the condition, and includes a reduction in the rate of progress, a halt in the rate of progress,

amelioration of the condition, and cure of the condition. Treatment as a prophylactic measure (i.e., prophylaxis) is also included.

"Treatment" also includes combination treatments and therapies, in which two or more treatments or therapies are combined, for example, sequentially or simultaneously.

For example, a therapeutically effective amount of a compound of formula I could be combined with radiation therapy or chemotherapy in the treatment of cancer.

The term "therapeutically-effective amount" as used herein, pertains to that amount of an active compound, or a material, composition or dosage form comprising an active compound, which is effective for producing some desired therapeutic effect, commensurate with a reasonable benefit/risk ratio.

It will be appreciated by one of skill in the art that appropriate dosages of the compounds, and compositions comprising the compounds, can vary from patient to patient. Determining the optimal dosage will generally involve the balancing of the level of therapeutic benefit against any risk or deleterious side effects. The selected dosage level will depend on a variety of actors including, but not limited to, the activity of the particular compound, the route of administration, the time of administration, the rate of excretion of the compound, the duration of the treatment, other drugs, compounds, and/or materials used in combination, the severity of the condition, general health, and prior medical history of the patient.

The invention also provides a method of targeting PNA oligomers to the cytoplasm of a cell using a lipophilic cation conjugate of Formula I, said method comprising delivering the PNA oligomers across the plasma membrane, without promoting selective aggregation in the mitochondria of said cell.

The invention will now be described in more detail with reference to the following non-limiting experimental section.

EXPERIMENTAL

Methods

Chemical synthesis of bisthiobutyltriphenylphosphonium (bisTBTP)

Thiobutyltriphenylphosphonium (TBTP) was generated by base hydrolysis of acylated TBTP as described (Burns *et al.*, 1995). Equal volumes of 1 M NaOH and 500 mg acylated TBTP dissolved in 95% ethanol were mixed and incubated for 20 minutes at room temperature, then diluted (1:40) in 150 mM HEPES, pH 7.3. The solution of TBTP at pH 7.3 was incubated with 0.2 g diamide ($(CH_3)_2NCON{=}NCON(CH_3)_2$, Sigma) for 1 h at room temperature. The formation of bisTBTP was followed by the disappearance of free thiols assayed as described in the thiol assay section below. After quenching with 1 M HCl (0.5 vol.), 0.5 g NaBr was added to ensure a Br^- counterion. The bisTBTP was extracted into 1 vol. dichloromethane three times, leaving unreacted diamide in the aqueous phase. The bisTBTP was precipitated from the dichloromethane by addition of diethyl ether (50 mL) giving a white powder (244 mg, 48% yield). The identity of bisTBTP was determined by ^1H NMR spectroscopy in $CDCl_3$, acquired using a Varian Gemini 200 MHz spectrophotometer at 25°C.

The analysis gave the following peaks: δ 7.6-8.0 (30 H, m, ($\mathbf{Ph_3}P\text{-}CH_2\text{-}CH_2\text{-}CH_2\text{-}CH_2)_2S_2$), δ 3.92 (4H, b, ($Ph_3P\text{-}\mathbf{CH_2}\text{-}CH_2\text{-}CH_2\text{-}CH_2)_2S_2$), δ 2.80 (4H, t, J = 7.2 H_2 ($Ph_3P\text{-}CH_2\text{-}CH_2\text{-}CH_2\text{-}\mathbf{CH_2})_2S_2$), δ 2.06-2.11 (4H, m, ($Ph_3P\text{-}CH_2\text{-}CH_2\text{-}\mathbf{CH_2}\text{-}CH_2)_2S_2$), δ 2.09 (4H, m, ($Ph_3P\text{-}CH_2\text{-}\mathbf{CH_2}\text{-}CH_2\text{-}CH_2)_2S_2$) δ 1.74 (4H, m, ($Ph_3P\text{-}CH_2\text{-}CH_2\text{-}\mathbf{CH_2}\text{-}CH_2)_2S_2$). Trace amounts of impurity detected by NMR had chemical shifts corresponding to diethyl ether (δ 1.19-1.25, 3.47 and 3.49). The final product was stored at -20°C. The identity of bisTBTP was further confirmed by base hydrolysis with 1 M NaOH which exposed 1.9 mol sulfhydryl per mol bisTBTP quantitated by the free thiol assay described below.

Thiol assay

The coupling efficiency of thiol containing compounds was monitored by assaying free thiol groups. Thiol groups react with 5,5'-dithiobis(2-nitrobenzoic acid) (DTNB) to form 5-thio-2-nitrobenzoic acid (TNB) that has a strong absorbance at 412 nm (ε_{412}=

$13.6 \times 10^3\ M^{-1}\ cm^{-1}$; (Ellman and Lysko, 1979)). The stoichiometry of the reaction is 1:1 and therefore the formation of TNB is proportional to the free thiol concentration. The total thiol content was estimated by adding 20 µL sample to 980 µL buffer containing 80 mM $NaHPO_4$ (pH 8.0, NaOH) and A_{412} was read before initiating the reaction by addition of 20 µL DTNB (10 mM DTNB in 0.1 M $NaHPO_4$, pH 8.0) and again 20 minutes later and the difference in absorbance used to calculate the thiol content. To correct for the absorbance due to degraded DTNB, the A_{412} of a buffer sample to which 20 µL DTNB was added was subtracted from the final absorbance.

Chemical synthesis of disulfide linked TPP--bioPNA conjugates

Peptide nucleic acids (PNA) targeting a unique region in both the human and mouse *PAX2* mRNA (Acc. No. NM_003989.1 and X55781.1,respectively) were synthesised by Applied Biosystems Inc. (Bedford, MA). The oligomers used were: Fluoroscein-XX-TTCACACCCCCGTGCC-X-Cys-CO_2H and Biotin-XX-GTTGGCTCTCT-X-Cys-CO_2H, where X is 8-amino-3,6-dioxanoic acid. To conjugate PNA to triphenylphosphonium cations, PNA oligomers (50 nmol) in 50 µL 10 mM HEPES, 1 mM EDTA, pH 7.5 were incubated with a non-thiol containing reducing agent, Tris[2-carboxyethyl]phosphine hydrochloride (TCEP·HCl), 2 equiv., at 37°C for 1 h. Then bisTBTP (5 equiv.) in 20 µL 10 mM HEPES, 1mM EDTA, pH 7.5 was added and incubated at 37°C for a further 4 h. The reaction products were separated by RP-HPLC on a C_4 analytical column (Vydac, 300 Å, 4.6 mm x 250 mm), using a Waters 450 HPLC system and a linear gradient from 0.1% TFA in water to 90% acetonitrile and 0.1% TFA was run over 30 minutes. Disulfide linked TPP-bioPNA conjugate peaks were detected by absorbance at 260 nm, collected, lyophilised and dissolved in water for further analysis.

The major peak at ~ 16 min (shown by an asterisk in Fig 3A), due to the TPP-bioPNA conjugate, was collected, lyophilized, dissolved in water and a sample analysed by RP-HPLC (Fig 3B). (Fig 3C shows purification of TPP-fluPNA by RP-HPLC. The peak at ~ 16 min (shown by an asterisk) is that of the TPP-fluPNA conjugate and this was collected and a sample analysed by RP-HPLC (Fig 3D).

The concentration of the TPP-PNA conjugates was determined at 55°C using the cumulative extinction coefficients of unmodified PNA (97,900 $M^{-1}.cm^{-1}$) and TBTP (2,500 $M^{-1}.cm^{-1}$), to give a value of 100,400 $M^{-1}.cm^{-1}$. The extinction coefficient for TPP-fluPNA is 158,500 $M^{-1}.cm^{-1}$.

MALDI ToF mass spectrometry

PNA conjugates (~0.5 pmol) in water were mixed with 3,5-dimethoxy-4-hydroxycinnamic acid (~0.5 µL of a 10 $mg.mL^{-1}$ solution) and after crystalisation were analysed by Matrix Assisted Laser Desorption/Ionization Time of Flight Mass Spectrometry (MALDI ToF MS) using a Finnigan MAT Lasermat 2000 instrument. Spectra were acquired in positive ion mode using melittin (M_w 2,846 Da) as an external mass calibrant.

MALDI ToF analysis of purified TPP-PNAs is shown in Fig 4A and 4B). The observed mass for the TPP-bioPNA was 4090.7 Da, within 0.01% of the calculated mass (4091.8 Da), as expected for external mass calibration (Fig 4A). The observed mass for the TPP-fluPNA was 5313.19 Da, within 0.1% of the calculated mass (4962.74 Da) (Fig 4B).

Cell culture and incubations with PNA

Cells (143B, human fiboblasts, COS-7, P388 and IMCD) were grown at 37°C and 5% CO_2 in humidified atmosphere in Dulbecco's modified Eagle's medium supplemented with 10% inactivated foetal calf serum (FCS). All cell culture media contained 100 $units.mL^{-1}$ penicillin, and 100 $\mu g.mL^{-1}$ streptomycin. Protein was quantitated by the bicinchoninic acid assay. Cells were incubated with 1 µM TPP-PNA or with PNA alone for the indicated times. The disulfide bond in the TPP-PNA is stable in the oxidizing extracellular environment but is labile in the reducing cytoplasmic mulieu (Fig 1).

143B cell incubations

For incubations in suspension 143B cells were harvested using trypsin and 10^6 cells were suspended in 1 mL DMEM, 10 mM HEPES, pH 7.0 and 10% FCS. For cell subfractionation, 143B cells were grown to confluence in 24 well tissue culture plates overnight and then incubated with 1 μM TPP-PNA conjugates ± 10 μM FCCP for 1 h at 37°C and after washing the cells were harvested by scraping in 250 mM sucrose, 20 mM MOPS, 3 mM EDTA, pH 6.7, and 1 $mg.mL^{-1}$ digitonin. A mitochondria enriched fraction was prepared from 200 μL crude suspension by centrifugation (10,000 x *g*, 1 min) through 300 μL oil (58% silicone oil (Dow Corning)/ 42% dioctyl phthalate) into 100 μL 0.5 M sucrose/0.1% Triton X-100, leaving a cytoplasm enriched upper layer. About 92 - 96% of total citrate synthase (Srere, 1969) and 0.3 - 1% lactate dehydrogenase activities (Berry *et al.*, 1991) were found in the mitochondria enriched fraction. Both fractions were immunoblotted to detect TPP-PNA conjugate localisation.

Gel electrophoresis and immunoblotting

Immunoblotting of TPP-PNA conjugates is shown in Fig 4C. Serial dilutions of the TPP-PNA conjugates were absorbed on nitrocellulose and the triphenylphosphonium moiety detected using anti triphenylphosphonium serum. BSA conjugated to IBTP (~1 μg protein) was used as a positive control. Horse radish peroxidase conjugated to extravidin was used to detect biotin and the bioPNA oligomer (~5 nmol) was used as a positive control.

For conjugate detection, 143B cell lysates treated with TPP-PNA conjugates (~5 nmol) in 20 μL loading buffer (50 mM Tris, 4% SDS, 12% glycerol, 2% 2-mercaptoethanol, 0.01% coomassie brilliant blue) were separated on 18.5% Tris-tricine gels using a BioRad Mini Protean system (Schagger and von Jagow, 1987). For *PAX-2* detection, P388 and IMCD cell lysates in loading buffer were resolved on 12.5 % Tris-glycine gels (Laemmli, 1970). Gels were then either fixed and stained with coomassie brilliant blue [0.1% (w/v) coomassie brilliant blue R-250, 45% (v/v) methanol and 10% (v/v) acetic acid], or electrotransfered onto 0.2 μm nitrocellulose using a BioRad Mini Trans-

Blot system (100 V, 1h) in transfer buffer (25 mM Tris-HCl, pH 8.3, 192 mM glycine, 20% methanol) and then blocked with 2% (w/v) fat-free milk powder in TBS (5 mM Tris.HCl, pH 7.4, 20 mM NaCl), 0.1% Tween-20. Rabbit anti-PAX2 polyclonal antibody (Zymed) diluted 1:1000 in TBS, 0.1% fat free milk powder, 0.1% Tween-20 was used for *PAX-2* detection following overnight incubation with the membrane. After 3 x 10 min washes in TBS, 0.1% Tween-20, horseradish peroxidase conjugated goat antirabbit IgG (1:10,000, Biorad) was used as a secondary antibody. To detect biotin, horse radish peroxidase conjugated extravidin (1:3,000, Sigma) was used. In both cases secondary antibody binding was carried out for 1 h at room temperature, followed by 3 x 10 min washes in TBS and visualized by chemiluminescence using a Pierce Super Signal R chemiluminescence substrate with Kodak X-OMATTM AR imaging film.

Fluorescence microscopy

For immunocytochemistry trypsinised human fibroblasts (5,000 cells per well) were plated onto 13 mm diameter glass coverslips in 24 well plates overnight. Following incubation for 4 h at 37°C with 1 μM TPP-PNA conjugates, cells were fixed with 4% paraformaldehyde (PFH) in TBS for 30 min, washed with TBS and incubated with 10 % FCS/0.1% Triton X-100/TBS (TBST) for 10 min. The IgG fraction of anti-triphenylphosphonium serum (1:500) was diluted in TBST, then added and incubated overnight at 4°C. The IgG fraction of preimmune serum was used as a control. After washing with TBS (3 x 5 min) the cells were incubated with anti-rabbit IgG Oregon green fluorophore-conjugated secondary antibody diluted 1:100 in TBS for 15 min in the dark. The cells were washed in TBS and incubated for another 15 min with streptavidin conjugated CY3 (1:200, Molecular Probes) in the dark, to detect biotin. Cells were then washed in TBS, mounted in DABCO/PVA medium (15 g PVA, 15 g 1,4-diazabicyclo (2,2,2) octane in 30% glucerol in 0.1 M Tris, pH 8.5) and mounted onto coverslips. Images were acquired using a BioRad MRC 600 laser-scanning confocal microscope using a Nikon Diaphot TMD inverted microscope and Nikon x60 NA 1.4 oil immersion Plan-Apochromat objective. The 568 nm and 488 nm lines of a Krypton-Argon laser and K1/K2 filter blocks were used at identical gain, black settings and time frame.

For real time fluorescence microscopy cells were grown in 35 mm diameter dishes overnight until they reached 80% confluence. Following incubation for 1 h at 37°C with 1 μM disulfide linked TPP-fluPNA conjugates the cells were incubated with 25 nM concentrations of MitoTracker Red (Molecular Probes) for 30 min at 37°C. The cells were then washed in TBS (3 x 10 min) and fresh DMEM media was added. Images were acquired using a Zeiss inverted confocal microscope with a Plan-Neofluar 40x/1.3 oil DIC objective, line 488 nm and Zeiss Imaging Software with equal exposure times.

Results

Synthesis and characterisation of TPP-PNA conjugates

The synthesis of the TPP-PNA conjugate was carried out in two stages outlined in Fig 2. The first stage was the synthesis of bisthiobutyl-triphenylphosphonium (bisTBTP) that would then link to thiol-containing compounds by a disulfide bond to a triphenylphosphonium cation. In the second stage the bisTBTP was reacted with a thiol containing PNA oligomers to form the TPP-PNA conjugate.

The TPP-PNA conjugates were purified by RP-HPLC and analysed by immunoblotting against the phosphonium moiety and against the biotin tag on one of the PNA oligomers. The identity of the TPP-PNA conjugates was confirmed by MALDI ToF mass spectrometry. To show that there were disulfide bonds present in both bisTBTP and in TPP-PNA, the compounds were base hydrolysed and the resulting free thiols quantitated (data not shown). The delivery of the PNA oligomer to the cytoplasm using the TPP-PNA conjugate was determined by western blotting and immunofluorescent confocal microscopy.

Synthesis

The lipophilic cation TBTP is a thiol reagent that is selectively directed to the mitochondrial matrix driven by the membrane potential (Fig 2). TBTP has a lipophilic

core, and a four carbon chain at the end of which is a thiol group to enable thiol-disulfide exchange.

TBTP has a protecting acyl group on the thiol to prevent oxidation during synthesis and storage. After deprotection of the acyl-TBTP by base hydrolysis the solution was adjusted to neutral pH and treated with the thiol-oxidising agent diamide. To synthesise bisthiobutyltriphenylphosphonium (bisTBTP) from monomeric TBTP, diamide stoichiometrically oxidises thiols to disulfides by the following reaction:

$(CH_3)_2NCON{=}NCON(CH_3)_2 + 2\ TBTP \Rightarrow (CH_3)_2NCONHNHCON(CH_3)_2 + bisTBTP$ (1)

Diamide oxidised TBTP to bisTBTP within 30 seconds seen by the rapid disappearance of free thiols on addition of diamide (data not shown). The reaction was left for an hour to ensure complete oxidation and the reaction was then quenched with acid. The bisTBTP was extracted from the diamide with dichloromethane and precipitated as a white powdery solid with a yield of about 50%. The identity of bisTBTP was confirmed by ^1H NMR. The shift in resonance of the peaks of 4 H are diagnostic of protons adjacent to a disulfide bond and indicated the presence of a disulfide link that was absent in TBTP. Base treatment of bisTBTP completely hydrolysed the disulfide bond releasing 1.90-2.0 mol free TBTP per mol of bisTBTP as detected by the thiol assay, and free thiols were not detected in bisTBTP (data not shown).

The PNA sequence was designed to target a unique region in both the human and mouse *PAX-2* mRNA and had a cysteine amino acid at the 3' end and a fluoroscein tag at the 5' end. A second PNA had a cysteine amino acid at the 3' end and a biotin tag at the 5' end. The triphenylphosphonium and biotin moieties enabled their detection by immunohistochemistry using either an anti-phosphonium antibody, or with a streptavidin-linked enzyme, respectively. The fluoroscein tag was used to follow TPP-PNA uptake in live cells by fluorescence microscopy. The disulfide linked TPP-PNA conjugates were formed through a disulfide exchange reaction of the free thiol from the cysteine with bisTBTP. The TPP-PNA conjugate was formed within an hour at pH 7.5.

Purification and characterisation of the TPP-PNA conjugate

The TPP-PNA conjugates were purified by RP HPLC (Fig. 3). The hydrophobic nature of the triphenylphosphonium moiety caused the conjugate to bind with higher affinity to the stationary phase, and to elute later than the unmodified PNA. The excess TBTP eluted latest due to its hydrophobicity (Fig. 3 A and C). A second HPLC run of the TPP-PNA fraction under the same conditions gave a single peak confirming the purity of the conjugate (Fig. 3 B and D). The identity of the conjugates was confirmed by MALDI TOF mass spectrometry which gave the expected molecular weights (Fig. 4 A and B). The composition of the TPP-PNAs was further characterised by immunoblotting (Fig. 4 C). The conjugates were adsorbed onto nitrocellulose, and probed for the triphenylphosphonium moiety using the cognate antiserum (Fig. 3 G). The biotin prosthetic group was detected by streptavidin binding conjugated to a horse radish peroxidase enzyme (Fig. 4 C). The antibody and the HRP enzyme could detect low amounts of the TPP-PNA conjugate (Fig. 4 C).

In summary, the TPP-PNA conjugates were synthesised, purified by RP HPLC and characterised by mass spectrometry and immunoblotting. This technique can be applied easily to other PNA oligomers to form a range of triphenylphosphonium-linked PNA conjugates.

Uptake of TPP-PNA by cells

Two techniques were used to analyze the efficiency of delivery of the TPP-PNA conjugates to the cytoplasm of cells in culture. The first was western blotting of mitochondrial and cytosolic fractions separated from conjugate-treated cells by homogenation followed by centrifugation. The second technique, fluorescent microscopy, used a fluorophore-conjugated antibody specific for the anti-triphenylphosphonium antibody and a streptavidin-linked fluorophore to detect the biotin tag on the PNA. In addition live cells incubated with the fluoresceinPNA and stained with a mitochondria-specific dye were used to follow the uptake and distribution of the *PAX2* PNA.

Western blotting following incubation of TPP-PNA with 143B and P388 cells

143B osteosarcoma cells (10^6) were incubated with 1 μM TPP-PNA in the presence or absence of $\Delta\Psi_m$, (± 10 μM FCCP) for 1 h at 37°C and the cells were then separated by homogenisation with digitonin (1 $mg.mL^{-1}$) in mitochondrial and cytosolic fractions (Figure 5A). These fractions were separated on non-reducing Tris-tricine gels, transferred onto nitrocellulose probed with streptavidin-linked horse radish peroxidase. PNA oligomers (5 nmol) were used as positive controls.

This procedure detected control PNA oligomer at the expected size (~3.2 kDa). Cells incubated with the TPP-PNA conjugate accumulated the PNA oligomer in the cytoplasm. The absence of a mitochondrial membrane potential by treatment with the mitochondrial uncoupler FCCP did not affect the cytoplasmic distribution of the PNA oligomers. It was possible to confirm the presence of the PNA in the cell fractions by comparing them to a control PNA sample.

These experiments were repeated with P388 cells which confirmed the localisation of the PNA within the cytoplasm of cells independent of the mitochondrial membrane potential (Fig. 5 B).

The P388 cells were treated as described before, the lysates were resolved on Tris-tricine gels and the TPP-fluPNA conjugates were detected using a GelDoc fluorescence imager.

Cells treated with the TPP-PNA conjugate rapidly (within 30 min) accumulated large amounts of the PNA oligomer in the cytoplasm compared with unmodified PNAs (Fig 5 C). The uptake of unmodified PNAs in cells most likely occurred by endocytosis as that has previously been shown to lead to the slow uptake of PNAs by cells. To test whether the phosphonium cation localized to mitochondria as a result of the large mitochondrial membrane potential, cytosolic fractions were probed using the anti-phosphonium serum (Fig 5 D). TBTP was detected in the mitochondrial fractions where it reacted with thiol-containing proteins and labeled them with a phosphonium cation. The absence of TBTP-bound proteins in the cytosolic fraction suggests that the cation is rapidly accumulated in mitochondria (Fig 5 D). These data indicate that TPP-

PNA is taken up by cells, reduced in the cytoplasmic environment, leaving the antisense PNA free to bind its target mRNA.

Confocal immunofluorescent microscopy of fibroblasts

The localisation of TPP-PNA conjugate within human fibroblasts was also determined by fixing cells that had been incubated with TPP-PNA, PNA or without any additions for up to 4 h and visualising the localisation of the PNA by confocal immunofluorescent microscopy (Fig. 6). Cells were incubated with 1 μM TPP-bioPNA at 37°C for 1 h (Fig 6A and 6C) and with 1 μM bioPNA for up to 4 h (Fig 6B and 6D).

Cells were fixed, incubated with antiserum against triphenylphosphonium (green) and a streptavidin-linked fluorophore to detect the biotin tagged PNA (red) and the images overlaid. (Fig 6B). Overlay of these two micrographs showed that the PNA was distributed throughout the cytoplasm, while the phosphonium was confined to the mitochondria (Fig 6A). This confirms that after delivery of the TPP-PNA to the cytoplasm it is reduced and the PNA remains in the cytoplasm, while the phosphonium cation is accumulated by the mitochondria. The unmodified PNA was also taken up by the cytoplasm and the nucleus but in far lower amounts (red) than the unmodified PNAs and only after a four hour incubation (Fig 6B).

Cells treated with bioPNA for 30 min, 1 h and 4 h were fixed and incubated with antiserum against triphenylphosphonium, streptavidin-linked fluorophore (red) and the nucleus was stained with 4, 6'-diamidino-2-phenylindole (DAPI, 3 $\mu g.mL^{-1}$) for 5 min (blue) to show the strict cytoplasmic localisation of the PNA (blue). Cells incubated with biotin PNA after showed uptake (red) only after 4 h. (Fig 6C)

Live cells were incubated with TPP-fluPNAs for 1 h and the mitochondria were stained with MitoTracker (25 nM) for 15 min. (D) Cells treated with fluorescein PNA for 1 h and stained with MitoTracker. Magnification, 1400 X. Scale bars, 20 μm.

Live cells treated with the ph-s-s-fluPNA conjugates confirmed cytoplasmic localisation of the PNA. In addition, these experiments show that the cation effectively

targeted at least 90% of the cells (Fig. 6C). Unmodified PNA accumulated very poorly into cells after an hour incubation (Fig. 6D).

These findings support the western blot data which also showed that low amounts of unmodified PNAs were taken up into the cytoplasm over a long incubation. In contrast the TPP-PNA delivery is both faster, and also delivers greater amounts of the PNA oligomers to the cytoplasm without trapping them in endosomes. Therefore the low $\Delta\Psi_p$ is sufficient to drive accumulation of TPP-PNA in the cytoplasm where the disulfide bond is rapidly reduced, releasing the PNA. This system should be useful to deliver PNAs to the cytoplasm in order to inhibit the transcription or translation of genes of interest within cells.

Down regulation of PAX2 protein expression using TPP-PNA conjugates

To investigate the biological effects of the anti-*PAX2* TPP-fluPNA conjugate we used a mouse cell line that highly expresses the *PAX2* protein. Treatment of P388 leukemia cancer cells with TPP-PNA conjugates resulted in a significant inhibition of *PAX2* expression that lasted 4 days (Fig 7). The knock down in *PAX2* expression was specific and did not affect general cell proliferation (Fig 7B). In contrast, treatment with unmodified anti-*PAX2* PNA did not decrease *PAX2* protein levels. This is most likely due to the absence of the cation that effectively delivers the PNA to the cells. Control experiments where cells were treated with media only showed the basal level of the *PAX2* protein over 4 days.

In summary, the triphenylphosphonium cation facilitates efficient delivery of the neutral PNAs to the cytoplasm of cells where they can inhibit the translation of *PAX2* mRNAs.

INDUSTRIAL APPLICABILITY

The strategy of targeting PNAs to the cytoplasm by conjugation to a triphenylphosphonium cation is an effective means of inhibiting gene expression. This conjugate delivery system has some advantages over conventional delivery methods: (i) the cation facilitates transport across the plasma membrane and is taken up into the

cytoplasm driven by the membrane potential, (ii) the disulfide bond is reduced in the cytoplasm, releasing the PNA, (iii) the uptake is rapid and occurs directly through the membrane without the need for receptor-mediated uptake or endo- or pinocytosis and (iv) the phosphonium uptake is indiscriminant of cell type. In addition, the conjugate is not cytotoxic in micromolar concentrations and is not degraded within the cell.

Unlike other antisense agents, PNAs can be used as antigene agents to target the DNA sequence of a gene through the formation of a triple-helix. This is potentially a more direct way to inhibit gene expression with fewer potential cellular targets than amplifiable mRNAs. Alternatively, as antisense agents, PNAs bind mRNAs more efficiently than any other available antisense agent (Knudsen *et al.*, 1996).

Delivery of PNAs to the cytoplasm by conjugation to disulphide linked lipophilic cations has the advantage of being simple, cheap and effective.

Triphenylphosphonium-linked delivery of PNAs may also be used as research tools. For example, with the assistance of cell array technology (Wu, 2000), they could be used to develop libraries of PNAs that could be delivered to cells for the purposes of drug discovery. Cell culture plates/slides containing spotted/arrayed individual TPP-PNAs (with thousands or hundreds of thousands of different sequences) could serve as a base for cells to be plated in such a way as to cover the arrayed TPP-PNAs like a blanket. The cells could contain a reporter of some sort, e.g., a gene promoter-reporter construct so as to assay for specific PNA sequences that disrupt a particular transcription factor's interaction with its target. The TPP-PNA would enter the cells located directly over the arrayed spot but wouldn't enter any other cells on the slide. The result of the uptake of the TPP-PNA into cells would be measured by the reporter inside the cells and detected in the array (on a spot-by-spot basis), using a scanning detector able to detect the signal from the reporter in the cells. This approach is not limited to analysis of effects of PNAs on gene activity. As the reporter could be anything giving a detectable signal, and need not be genetic in nature, the PNAs could influence reporter activity by non-genetic mechanisms, for example, by disrupting protein-protein interactions in the cytoplasm.

The TPP-PNA conjugates may be found that disrupt or modulate many kinds of cellular process through interaction with gene transcription mechanisms. For example, certain PNAs may be able to bind to DNA and prevent certain proteins from defining MARs (matrix attachment regions) during the early commitment of stem cells into cell lineages. This may have the effect of re-designing the chromatin packaging of a cell, and as a result the subsequent gene activity, thereby modulating how much, or what type of cells are determined in a tissue. This is possibly the mechanism by which some teratogenic agents act to cause birth defects.

It is to be understood that the scope of the invention is not limited to the examples described above and therefore that numerous variations and modifications may be made to the described embodiments without departing from the scope of the invention.

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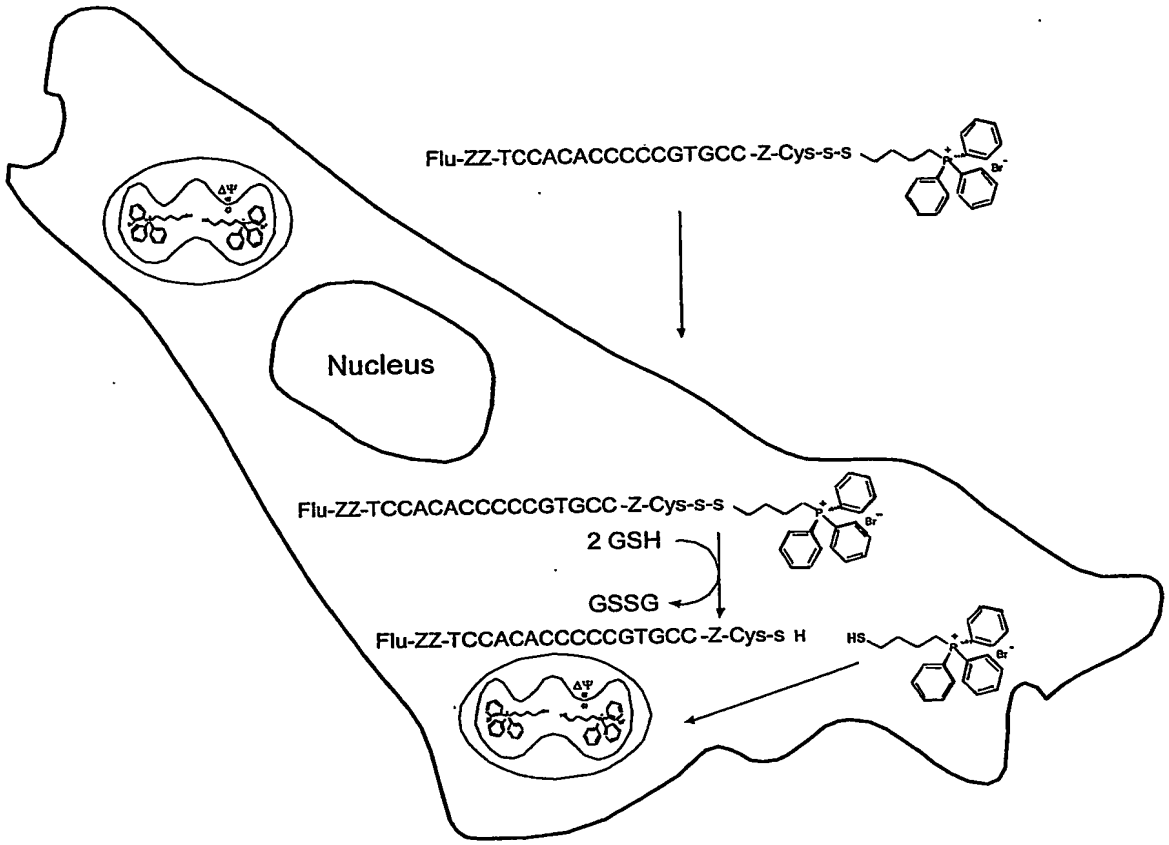
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Nucleus
Flu-ZZ-TCCACACCCCCGTGCC-Z-Cys-s-s
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GSSG
Flu-ZZ-TCCACACCCCCGTGCC-Z-Cys-s H
HS

FIGURE 1

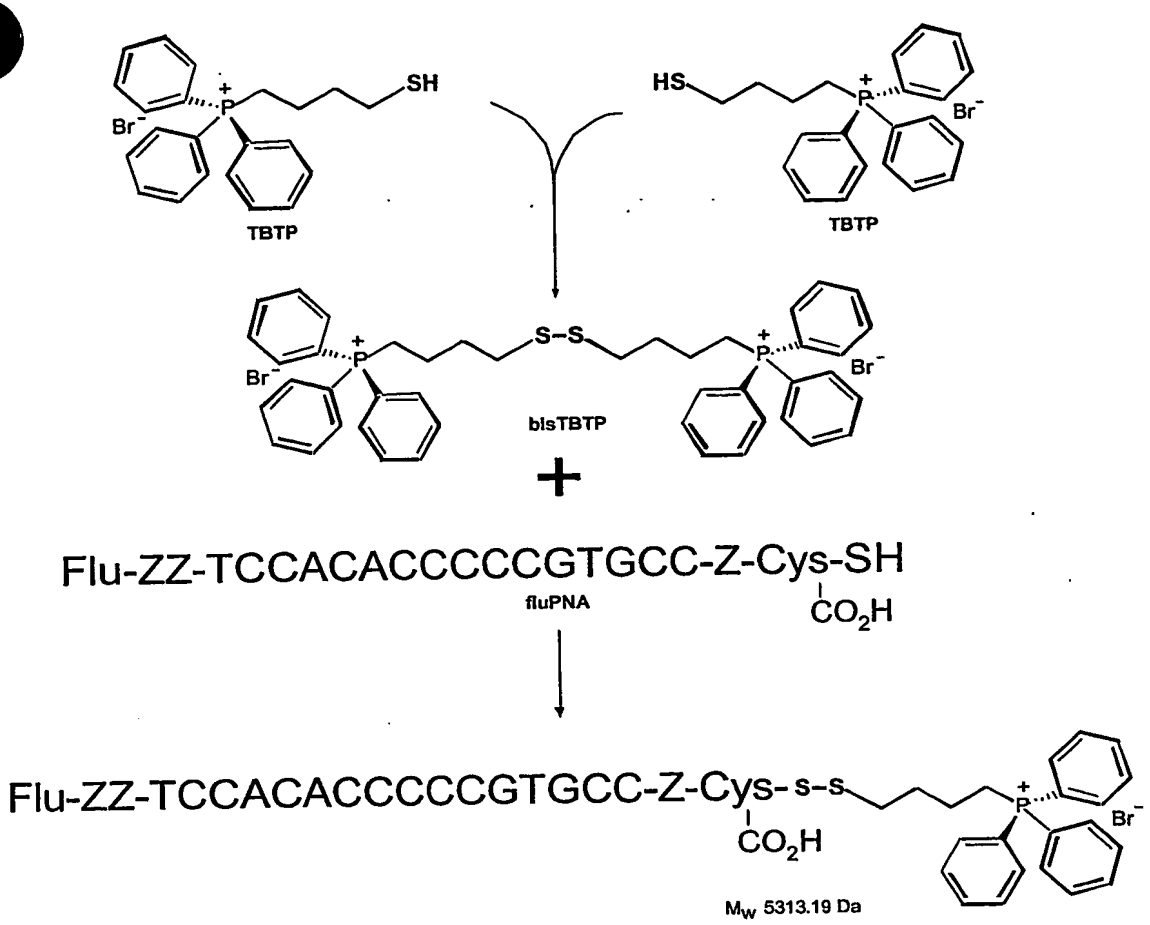
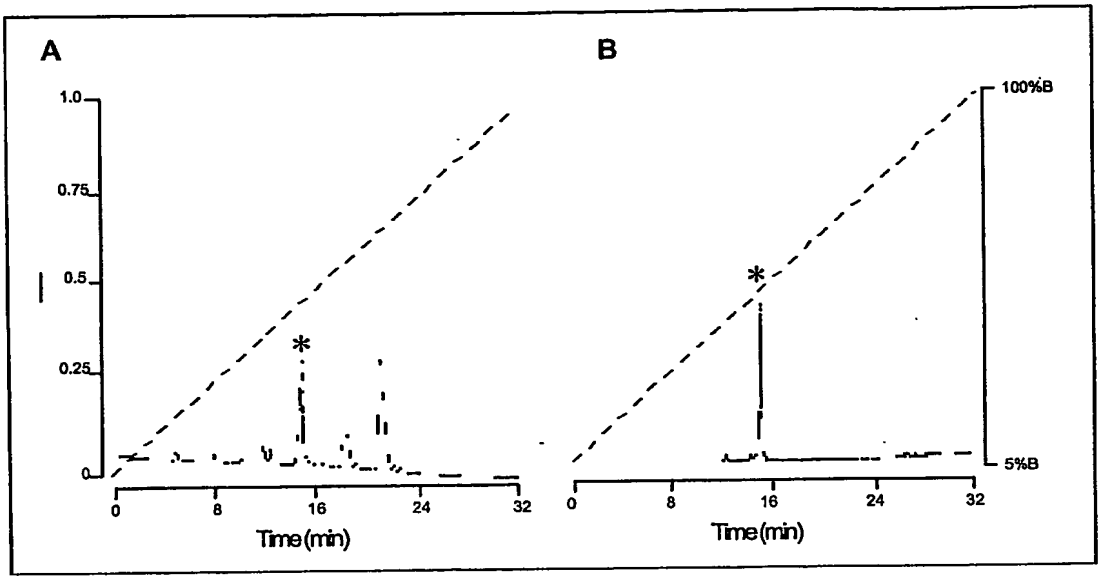

SH
HS
Br⁻
TBTP
TBTP
S–S
Br⁻
Br⁻
bisTBTP
+
Flu-ZZ-TCCACACCCCCGTGCC-Z-Cys-SH
fluPNA
CO2H
Flu-ZZ-TCCACACCCCCGTGCC-Z-Cys-
S–S
CO2H
Br⁻
Mw 5313.19 Da

FIGURE 2


A
B
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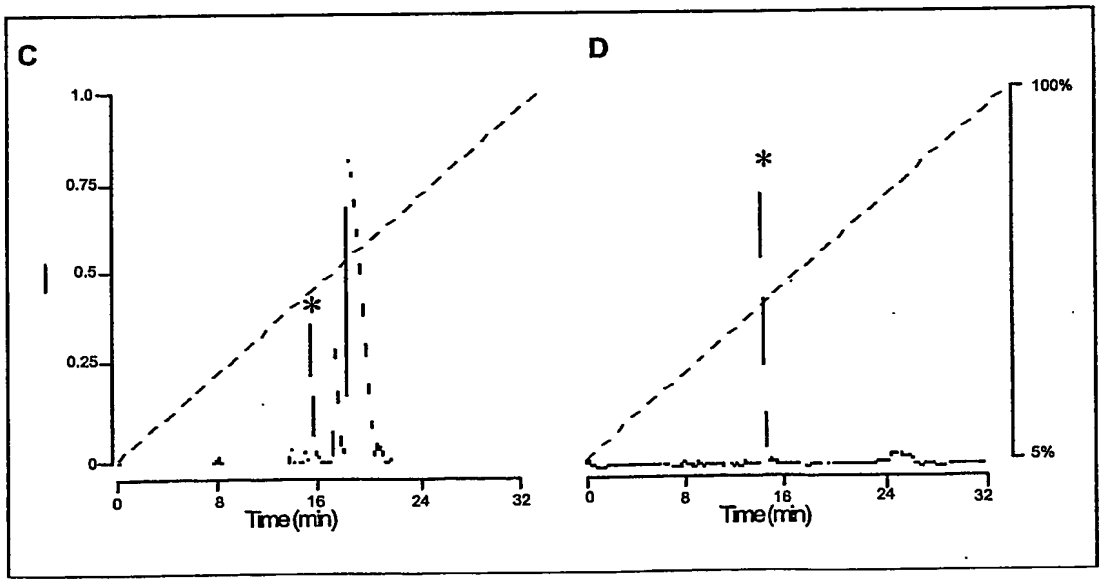
0.5
0.25
0
100%B
5%B
0
8
16
24
32
Time (min)


C
D
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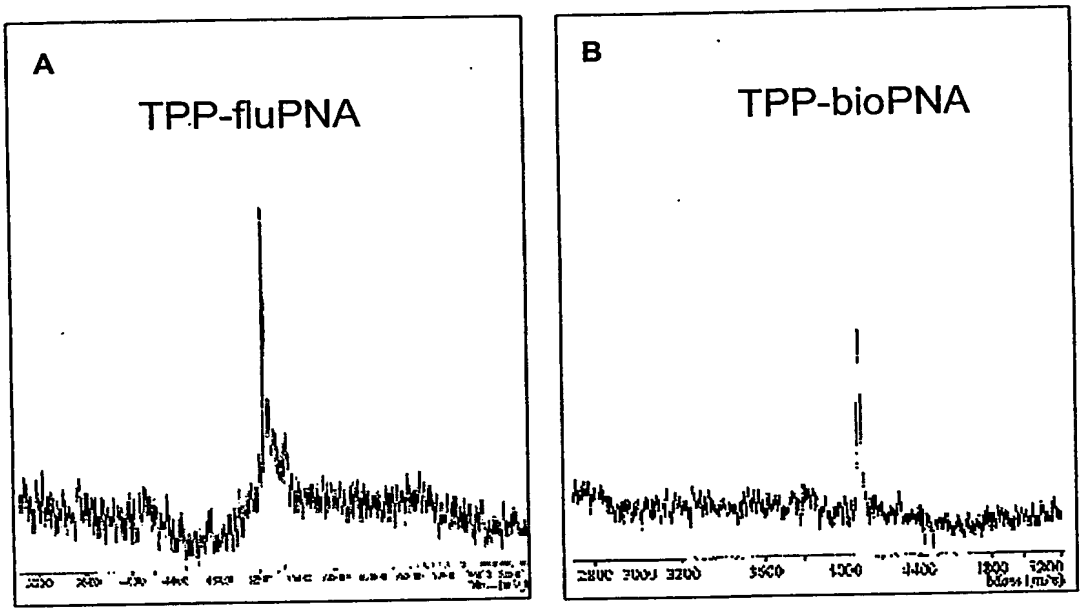
0.5
0.25
0
100%
5%
0
8
16
24
32
Time (min)

FIGURE 3



C

Amount (pmol)	2000	200	20	2	Control	Detection
TPP-bioPNA						TPP
TPP-bioPNA						Biotin

FIGURE 4

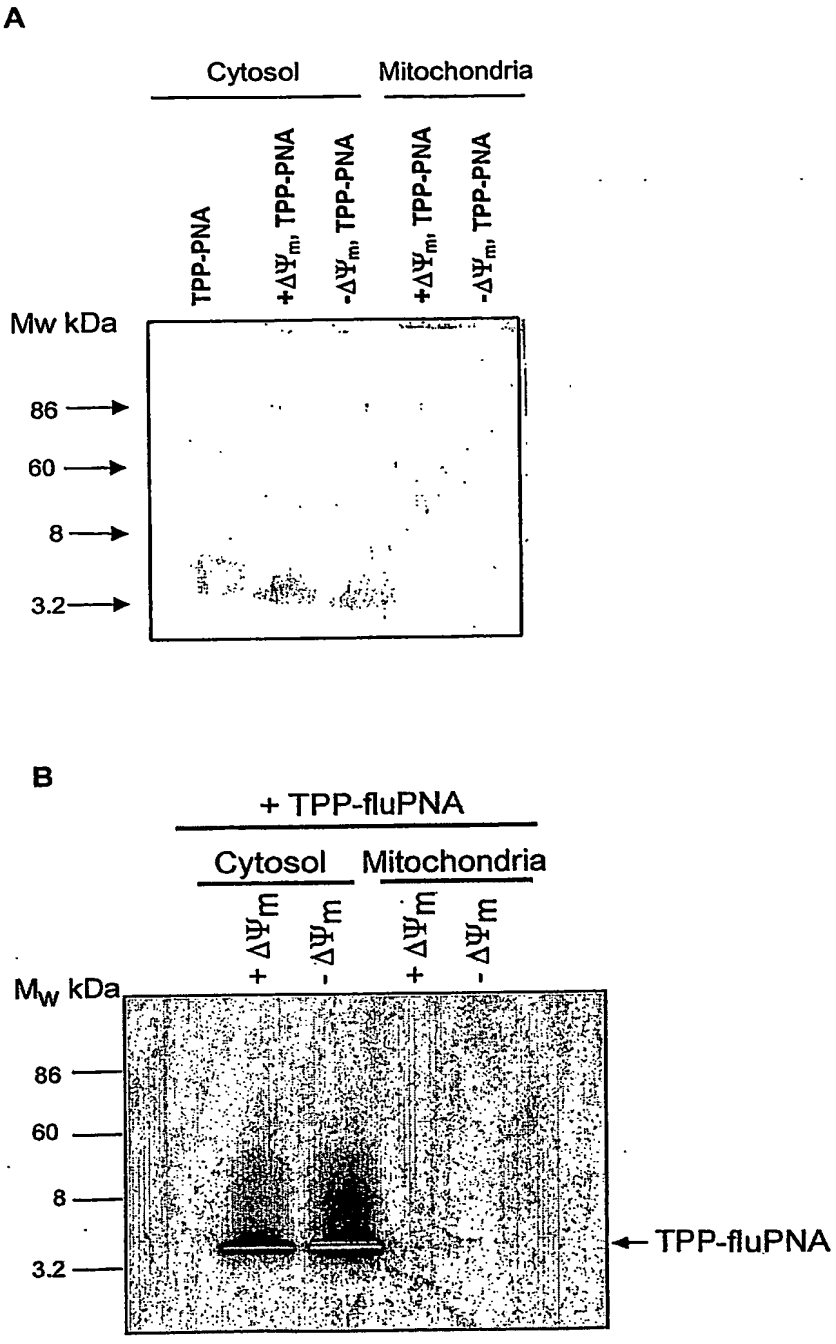
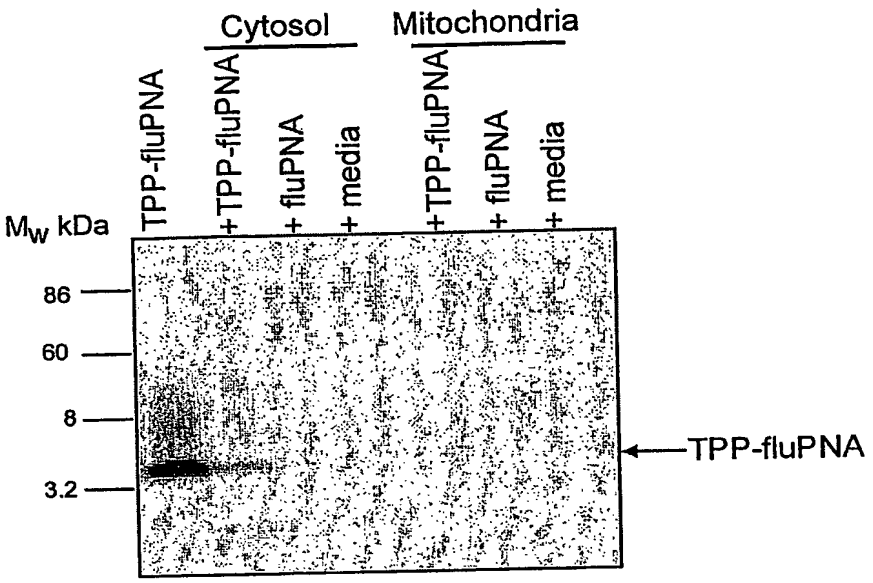

A
Cytosol
Mitochondria
TPP-PNA
+ΔΨm, TPP-PNA
-ΔΨm, TPP-PNA
+ΔΨm, TPP-PNA
-ΔΨm, TPP-PNA
Mw kDa
86
60
8
3.2
B
+ TPP-fluPNA
Cytosol
Mitochondria
+ ΔΨm
- ΔΨm
+ ΔΨm
- ΔΨm
MW kDa
86
60
8
3.2
TPP-fluPNA

FIGURE 5

C


Cytosol
Mitochondria
TPP-fluPNA
+TPP-fluPNA
+ fluPNA
+ media
+TPP-fluPNA
+ fluPNA
+ media
M_W kDa
86
60
8
3.2
TPP-fluPNA

D

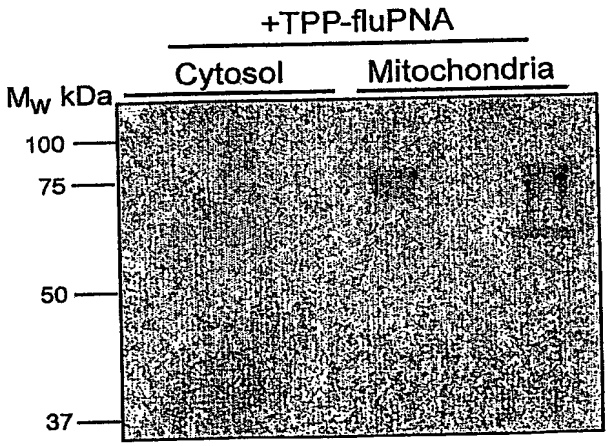

+TPP-fluPNA
Cytosol
Mitochondria
M_W kDa
100
75
50
37

FIGURE 5

Fig 6

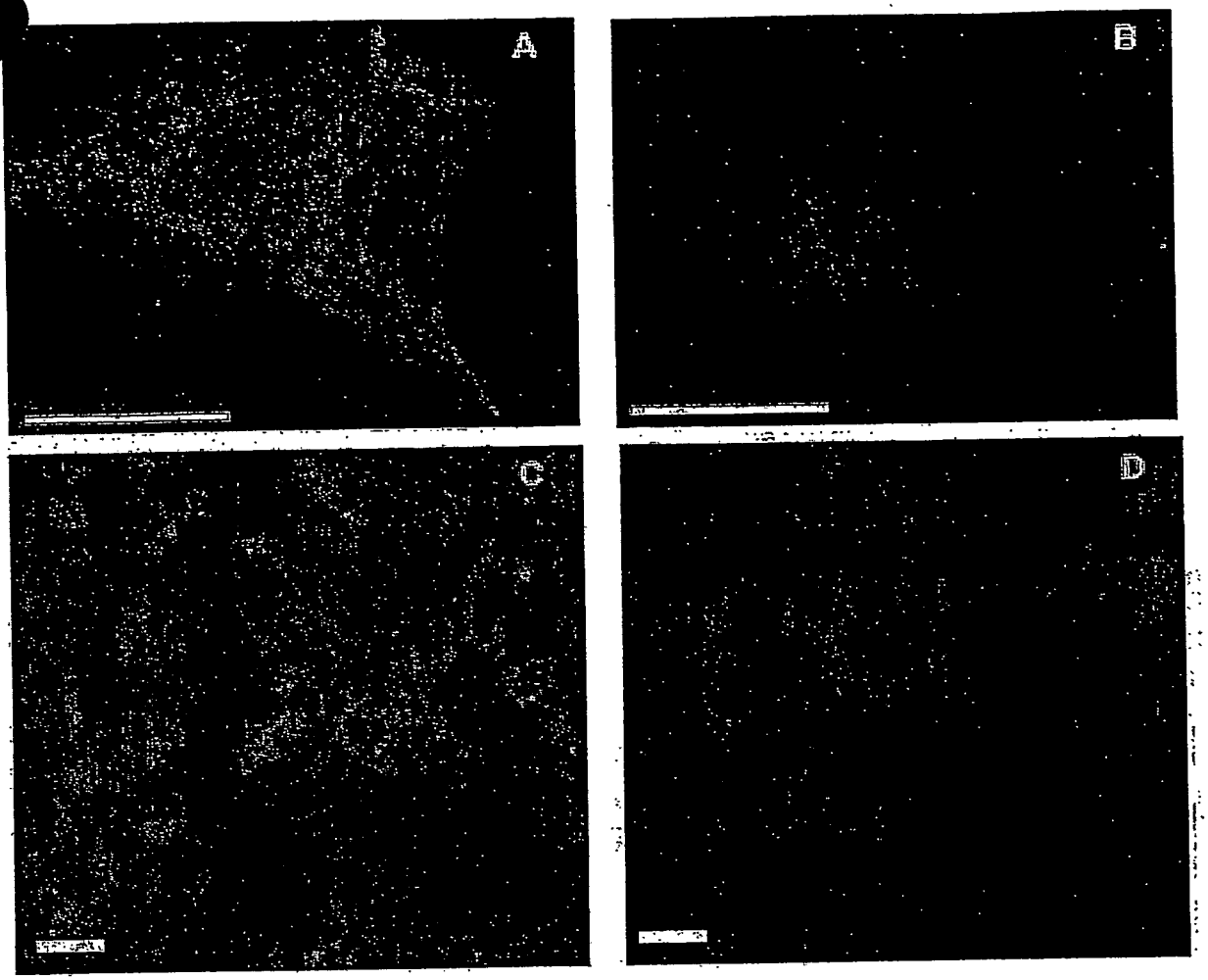


FIGURE 6

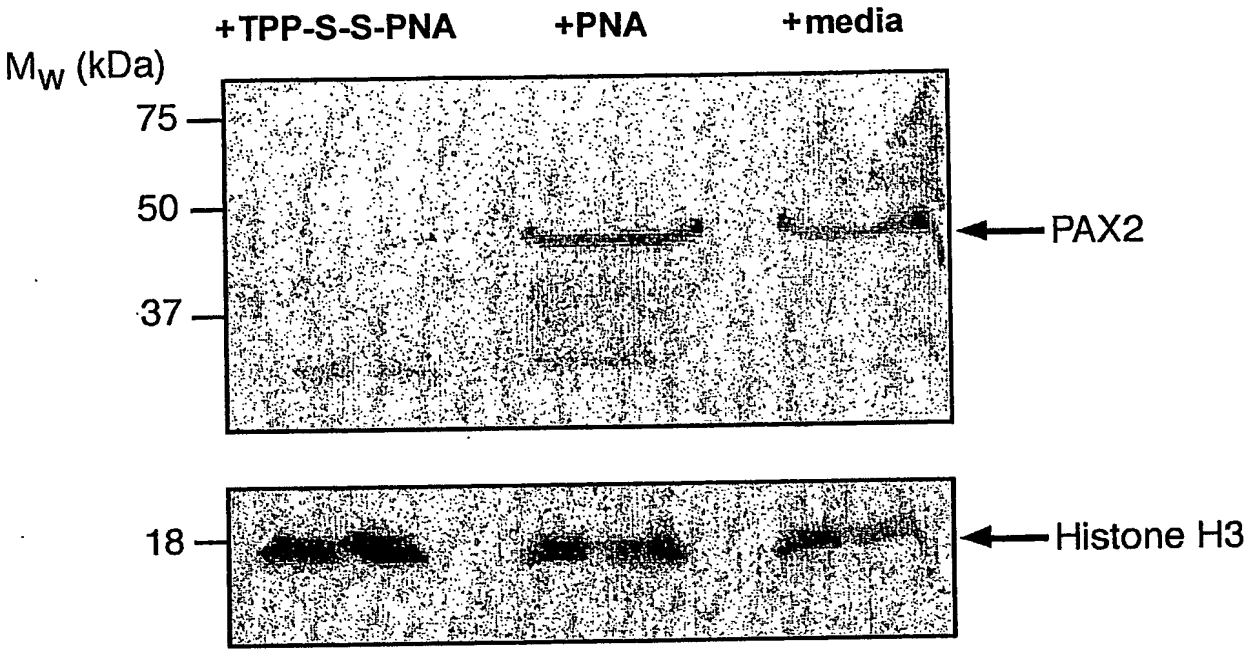


FIGURE 7